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of antigen. By synthesizing several catalyst molecules in close proximity, more efficient multistep conversions may be achieved by "coordinate immobilization." Coordinate immobilization also may be used for electron transfer systems, as well as to provide both structural integrity and other desirable properties to materials such as lubrication, wetting, etc.

According to alternative embodiments, molecular biodistribution or pharmacokinetic properties may be examined. For example, to assess resistance to intestinal or serum proteases, polymers may be capped with a fluorescent tag and exposed to biological fluids.

### III. Polymer Synthesis

FIG. 1 illustrates one embodiment of the invention disclosed herein in which a substrate 2 is shown in cross-section. Essentially, any conceivable substrate may be employed in the invention. The substrate may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, etc. The substrate may have any convenient shape, such as a disc, square, sphere, circle, etc. The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which the synthesis takes place. The substrate and its surface preferably form a rigid support on which to carry out the reactions described herein. The substrate and its surface is also chosen to provide appropriate light-absorbing characteristics. For instance, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO<sub>2</sub>, SiN<sub>4</sub>, modified silicon, or any one of a wide variety of gels or polymers such as (poly)tetrafluoroethylene, (poly)vinylidene difluoride, poly-styrene, polycarbonate, or combinations thereof. Other substrate materials will be readily apparent to those of skill in the art upon review of this disclosure. In a preferred embodiment the substrate is flat glass or single-crystal silicon with surface relief features of less than 10.

According to some embodiments, the surface of the substrate is etched using well known techniques to provide for desired surface features. For example, by way of the formation of trenches, v-grooves, mesa structures, or the like, the synthesis regions may be more closely placed within the focus point of impinging light, be provided with reflective "mirror" structures for maximization of light collection from fluorescent sources, or the like.

Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials. In some embodiments the surface may provide for the use of caged binding members which are attached firmly to the surface of the substrate. Preferably, the surface will contain reactive groups, which could be carboxyl, amino, hydroxyl, or the like. Most preferably, the surface will be optically transparent and will have surface Si—OH functionalities, such as are found on silica surfaces.

The surface 4 of the substrate is preferably provided with a layer of linker molecules 6, although it will be understood that the linker molecules are not required

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elements of the invention. The linker molecules are preferably of sufficient length to permit polymers in a completed substrate to interact freely with molecules exposed to the substrate. The linker molecules should be 6-50 atoms long to provide sufficient exposure. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing 2-10 monomer units, diamines, diacids, amino acids, or combinations thereof. Other linker molecules may be used in light of this disclosure.

According to alternative embodiments, the linker molecules are selected based upon their hydrophilic-/hydrophobic properties to improve presentation of synthesized polymers to certain receptors. For example, in the case of a hydrophilic receptor, hydrophilic linker molecules will be preferred so as to permit the receptor to more closely approach the synthesized polymer.

According to another alternative embodiment, linker molecules are also provided with a photocleavable group at an intermediate position. The photocleavable group is preferably cleavable at a wavelength different from the protective group. This enables removal of the various polymers following completion of the synthesis by way of exposure to the different wavelengths of light.

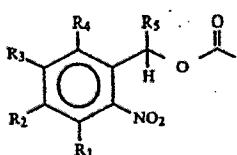
The linker molecules can be attached to the substrate via carbon-carbon bonds using, for example, (poly)trifluorochloroethylene surfaces, or preferably, by siloxane bonds (using, for example, glass or silicon oxide surfaces). Siloxane bonds with the surface of the substrate may be formed in one embodiment via reactions of linker molecules bearing trichlorosilyl groups. The linker molecules may optionally be attached in an ordered array, i.e., as parts of the head groups in a polymerized Langmuir Blodgett film. In alternative embodiments, the linker molecules are adsorbed to the surface of the substrate.

The linker molecules and monomers used herein are provided with a functional group to which is bound a protective group. Preferably, the protective group is on the distal or terminal end of the linker molecule opposite the substrate. The protective group may be either a negative protective group (i.e., the protective group renders the linker molecules less reactive with a monomer upon exposure) or a positive protective group (i.e., the protective group renders the linker molecules more reactive with a monomer upon exposure). In the case of negative protective groups an additional step of reactivation will be required. In some embodiments, this will be done by heating.

The protective group on the linker molecules may be selected from a wide variety of positive light-reactive groups preferably including nitro aromatic compounds such as o-nitrobenzyl derivatives or benzylsulfonyl. In a preferred embodiment, 6-nitroveratryloxycarbonyl (NVOC), 2-nitrobenzyloxycarbonyl (NBOC) or  $\alpha,\alpha$ -dimethyl-dimethoxybenzyloxycarbonyl (DDZ) is used. In one embodiment, a nitro aromatic compound containing a benzylic hydrogen ortho to the nitro group is used, i.e., a chemical of the form:

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where  $R_1$  is alkoxy, alkyl, halo, aryl, alkenyl, or hydrogen;  $R_2$  is alkoxy, alkyl, halo, aryl, nitro, or hydrogen;  $R_3$  is alkoxy, alkyl, halo, nitro, aryl, or hydrogen;  $R_4$  is alkoxy, alkyl, hydrogen, aryl, halo, or nitro; and  $R_5$  is alkyl, alkenyl, cyano, alkoxy, hydrogen, halo, aryl, or alkenyl. Other materials which may be used include  $\alpha$ -hydroxy- $\alpha$ -methyl cinnamoyl derivatives. Photoremoveable protective groups are described in, for example, Patchornik, *J. Am. Chem. Soc.* (1970) 92:6333 and Amit et al., *J. Org. Chem.* (1974) 39:192, both of which are incorporated herein by reference.

In an alternative embodiment the positive reactive group is activated for reaction with reagents in solution. For example, a 5-bromo-7-nitro indoline group, when bound to a carbonyl, undergoes reaction upon exposure to light at 420 nm.

In a second alternative embodiment, the reactive group on the linker molecule is selected from a wide variety of negative light-reactive groups including a cinamate group.

Alternatively, the reactive group is activated or deactivated by electron beam lithography, x-ray lithography, or any other radiation. Suitable reactive groups for electron beam lithography include sulfonyl. Other methods may be used including, for example, exposure to a current source. Other reactive groups and methods of activation may be used in light of this disclosure.

As shown in FIG. 1, the linking molecules are preferably exposed to, for example, light through suitable mask 8 using photolithographic techniques of the type known in the semiconductor industry and described in, for example, Sze, *VLSI Technology*, McGraw-Hill (1983), and Mead et al., *Introduction to VLSI Systems*, Addison-Wesley (1980), which are incorporated herein by reference for all purposes. The light may be directed at either the surface containing the protective groups or at the back of the substrate, so long as the substrate is transparent to the wavelength of light needed for removal of the protective groups. In the embodiment shown in FIG. 1, light is directed at the surface of the substrate containing the protective groups. FIG. 1 illustrates the use of such masking techniques as they are applied to a positive reactive group so as to activate linking molecules and expose functional groups in areas 10a and 10b.

The mask 8 is in one embodiment a transparent support material selectively coated with a layer of opaque material. Portions of the opaque material are removed, leaving opaque material in the precise pattern desired on the substrate surface. The mask is brought into close proximity with, imaged on, or brought directly into contact with the substrate surface as shown in FIG. 1. "Openings" in the mask correspond to locations on the substrate where it is desired to remove photoremoveable protective groups from the substrate. Alignment may be performed using conventional alignment techniques in which alignment marks (not shown) are used to accurately overlay successive masks with previous patterning steps, or more sophisticated techniques may be used. For example, interferometric techniques such as the one

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described in Flanders et al., "A New Interferometric Alignment Technique," *App. Phys. Lett.* (1977) 31:426-428, which is incorporated herein by reference, may be used.

To enhance contrast of light applied to the substrate, it is desirable to provide contrast enhancement materials between the mask and the substrate according to some embodiments. This contrast enhancement layer may comprise a molecule which is decomposed by light such as quinone diazid or a material which is transiently bleached at the wavelength of interest. Transient bleaching of materials will allow greater penetration where light is applied, thereby enhancing contrast. Alternatively, contrast enhancement may be provided by way of a cladded fiber optic bundle.

The light may be from a conventional incandescent source, a laser, a laser diode, or the like. If non-collimated sources of light are used it may be desirable to provide a thick- or multi-layered mask to prevent spreading of the light onto the substrate. It may, further, be desirable in some embodiments to utilize groups which are sensitive to different wavelengths to control synthesis. For example, by using groups which are sensitive to different wavelengths, it is possible to select branch positions in the synthesis of a polymer or eliminate certain masking steps. Several reactive groups along with their corresponding wavelengths for deprotection are provided in Table 1.

TABLE I

Group	Approximate Deprotection Wavelength
Nitroveratryloxy carbonyl (NVOC)	UV (300-400 nm)
Nitrobenzyl carbonyl (NBOC)	UV (300-350 nm)
Dimethyl dimethoxybenzyl carbonyl	UV (280-300 nm)
5-Bromo-7-nitroindolinyl	UV (420 nm)
$\alpha$ -Hydroxy- $\alpha$ -methyl cinnamoyl	UV (300-350 nm)
2-Oxymethylene anthraquinone	UV (350 nm)

While the invention is illustrated primarily herein by way of the use of a mask to illuminate selected regions the substrate, other techniques may also be used. For example, the substrate may be translated under a modulated laser or diode light source. Such techniques are discussed in, for example, U.S. Pat. No. 4,719,615 (Feyrer et al.), which is incorporated herein by reference. In alternative embodiments a laser galvanometric scanner is utilized. In other embodiments, the synthesis may take place on or in contact with a conventional liquid crystal (referred to herein as a "light valve") or fiber optic light sources. By appropriately modulating liquid crystals, light may be selectively controlled so as to permit light to contact selected regions of the substrate. Alternatively, synthesis may take place on the end of a series of optical fibers to which light is selectively applied. Other means of controlling the location of light exposure will be apparent to those of skill in the art.

The substrate may be irradiated either in contact or not in contact with a solution (not shown) and is, preferably, irradiated in contact with a solution. The solution contains reagents to prevent the by-products formed by irradiation from interfering with synthesis of the polymer according to some embodiments. Such by-products might include, for example, carbon dioxide, nitrosocarbonyl compounds, styrene derivatives, indole derivatives, and products of their photochemical reactions.

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Alternatively, the solution may contain reagents used to match the index of refraction of the substrate. Reagents added to the solution may further include, for example, acidic or basic buffers, thiols, substituted hydrazines and hydroxylamines, reducing agents (e.g., NADH) or reagents known to react with a given functional group (e.g., aryl nitroso + glyoxylic acid  $\rightarrow$  aryl formhydroxamate + CO<sub>2</sub>).

Either concurrently with or after the irradiation step, the linker molecules are washed or otherwise contacted with a first monomer, illustrated by "A" in regions 12a and 12b in FIG. 2. The first monomer reacts with the activated functional groups of the linkage molecules which have been exposed to light. The first monomer, which is preferably an amino acid, is also provided with a photoprotective group. The photoprotective group on the monomer may be the same as or different than the protective group used in the linkage molecules, and may be selected from any of the above-described protective groups. In one embodiment, the protective groups for the A monomer is selected from the group NBOC and NVOC.

As shown in FIG. 3, the process of irradiating is thereafter repeated, with a mask repositioned so as to remove linkage protective groups and expose functional groups in regions 14a and 14b which are illustrated as being regions which were protected in the previous masking step. As an alternative to repositioning of the first mask, in many embodiments a second mask will be utilized. In other alternative embodiments, some steps 25 may provide for illuminating a common region in successive steps. As shown in FIG. 3, it may be desirable to provide separation between irradiated regions. For example, separation of about 1-5  $\mu$ m may be appropriate to account for alignment tolerances.

As shown in FIG. 4, the substrate is then exposed to a second protected monomer "B," producing B regions 16a and 16b. Thereafter, the substrate is again masked so as to remove the protective groups and expose reactive groups on A region 12a and B region 16b. The substrate 40 is again exposed to monomer B, resulting in the formation of the structure shown in FIG. 6. The dimers B—A and B—B have been produced on the substrate.

A subsequent series of masking and contacting steps similar to those described above with A (not shown) 45 provides the structure shown in FIG. 7. The process provides all possible dimers of B and A, i.e., B—A, A—B, A—A, and B—B.

The substrate, the area of synthesis, and the area for synthesis of each individual polymer could be of any size or shape. For example, squares, ellipsoids, rectangles, triangles, circles, or portions thereof, along with irregular geometric shapes, may be utilized. Duplicate synthesis areas may also be applied to a single substrate for purposes of redundancy.

In one embodiment the regions 12a, 12b and 16a, 16b on the substrate will have a surface area of between about 1 cm<sup>2</sup> and 10<sup>-10</sup> cm<sup>2</sup>. In some embodiments the regions 12a, 12b and 16a, 16b have areas of less than about 10<sup>-1</sup> cm<sup>2</sup>, 10<sup>-2</sup> cm<sup>2</sup>, 10<sup>-3</sup> cm<sup>2</sup>, 10<sup>-4</sup> cm<sup>2</sup>, 10<sup>-5</sup> 60 cm<sup>2</sup>, 10<sup>-6</sup> cm<sup>2</sup>, 10<sup>-7</sup> cm<sup>2</sup>, 10<sup>-8</sup> cm<sup>2</sup>, or 10<sup>-10</sup> cm<sup>2</sup>. In a preferred embodiment, the regions 12a, 12b and 16a, 16b are between about 10  $\times$  10  $\mu$ m and 500  $\times$  100  $\mu$ m.

In some embodiments a single substrate supports more than about 10 different monomer sequences and 65 preferably more than about 100 different monomer sequences, although in some embodiments more than about 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, or 10<sup>8</sup> different sequences

are provided on a substrate. Of course, within a region of the substrate in which a monomer sequence is synthesized, it is preferred that the monomer sequence be substantially pure. In some embodiments, regions of the substrate contain polymer sequences which are at least about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% pure.

According to some embodiments, several sequences are intentionally provided within a single region so as to provide an initial screening for biological activity, after which materials within regions exhibiting significant binding are further evaluated.

#### IV. Details of One Embodiment of a Reactor System

FIG. 8A schematically illustrates a preferred embodiment of a reactor system 100 for synthesizing polymers on the prepared substrate in accordance with one aspect of the invention. The reactor system includes a body 102 with a cavity 104 on a surface thereof. In preferred embodiments the cavity 104 is between about 50 and 1000  $\mu$ m deep with a depth of about 500  $\mu$ m preferred.

The bottom of the cavity is preferably provided with an array of ridges 106 which extend both into the plane of the FIGURE and parallel to the plane of the FIGURE. The ridges are preferably about 50 to 200  $\mu$ m deep and spaced at about 2 to 3 mm. The purpose of the ridges is to generate turbulent flow for better mixing. The bottom surface of the cavity is preferably light absorbing so as to prevent reflection of impinging light.

A substrate 112 is mounted above the cavity 104. The substrate is provided along its bottom surface 114 with a photoremoveable protective group such as NVOC with or without an intervening linker molecule. The substrate is preferably transparent to a wide spectrum of light, but in some embodiments is transparent only at a wavelength at which the protective group may be removed (such as UV in the case of NVOC). The substrate in some embodiments is a conventional microscope glass slide or cover slip. The substrate is preferably as thin as possible, while still providing adequate physical support. Preferably, the substrate is less than about 1 mm thick, more preferably less than 0.5 mm thick, more preferably less than 0.1 mm thick, and most preferably less than 0.05 mm thick. In alternative preferred embodiments, the substrate is quartz or silicon.

The substrate and the body serve to seal the cavity except for an inlet port 108 and an outlet port 110. The body and the substrate may be mated for sealing in some embodiments with one or more gaskets. According to a preferred embodiment, the body is provided with two concentric gaskets and the intervening space is held at vacuum to ensure mating of the substrate to the gaskets.

Fluid is pumped through the inlet port into the cavity by way of a pump 116 which may be, for example, a model no. B-120-S made by Eldex Laboratories. Selected fluids are circulated into the cavity by the pump, through the cavity, and out the outlet for recirculation or disposal. The reactor may be subjected to ultrasonic radiation and/or heated to aid in agitation in some embodiments.

Above the substrate 112, a lens 120 is provided which may be, for example, a 2" 100 mm focal length fused silica lens. For the sake of a compact system, a reflective mirror 122 may be provided for directing light from a light source 124 onto the substrate. Light source 124 may be, for example, a Xe(Hg) light source manufactured by Oriel and having model no. 66024. A second lens 126 may be provided for the purpose of projecting

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a mask image onto the substrate in combination with lens 120. This form of lithography is referred to herein as projection printing. As will be apparent from this disclosure, proximity printing and the like may also be used according to some embodiments.

Light from the light source is permitted to reach only selected locations on the substrate as a result of mask 128. Mask 128 may be, for example, a glass slide having etched chrome thereon. The mask 128 in one embodiment is provided with a grid of transparent locations and opaque locations. Such masks may be manufactured by, for example, Photo Sciences, Inc. Light passes freely through the transparent regions of the mask, but is reflected from or absorbed by other regions. Therefore, only selected regions of the substrate are exposed to light.

As discussed above, light valves (LCD's) may be used as an alternative to conventional masks to selectively expose regions of the substrate. Alternatively, fiber optic faceplates such as those available from 20 Schott Glass, Inc, may be used for the purpose of contrast enhancement of the mask or as the sole means of restricting the region to which light is applied. Such faceplates would be placed directly above or on the substrate in the reactor shown in FIG. 8A. In still further embodiments, flys-eye lenses, tapered fiber optic faceplates, or the like, may be used for contrast enhancement.

In order to provide for illumination of regions smaller than a wavelength of light, more elaborate techniques 30 may be utilized. For example, according to one preferred embodiment, light is directed at the substrate by way of molecular microcrystals on the tip of, for example, micropipettes. Such devices are disclosed in Lieberman et al., "A Light Source Smaller Than the Optical 35 Wavelength," *Science* (1990) 247:59-61, which is incorporated herein by reference for all purposes.

In operation, the substrate is placed on the cavity and sealed thereto. All operations in the process of preparing the substrate are carried out in a room lit primarily 40 or entirely by light of a wavelength outside of the light range at which the protective group is removed. For example, in the case of NVOC, the room should be lit with a conventional dark room light which provides little or no UV light. All operations are preferably conducted at about room temperature.

A first, deprotection fluid (without a monomer) is circulated through the cavity. The solution preferably is of 5 mM sulfuric acid in dioxane solution which serves to keep exposed amino groups protonated and decreases 50 their reactivity with photolysis by-products. Absorptive materials such as N,N-diethylamino-2,4-(dinitrobenzene, for example, may be included in the deprotection fluid which serves to absorb light and prevent reflection and unwanted photolysis.

The slide is, thereafter, positioned in a light raypath from the mask such that first locations on the substrate are illuminated and, therefore, deprotected. In preferred embodiments the substrate is illuminated for between about 1 and 15 minutes with a preferred illumination time of about 10 minutes at 10-20 mW/cm<sup>2</sup> with 365 nm light. The slides are neutralized (i.e., brought to a pH of about 7) after photolysis with, for example, a solution of di-isopropylethylamine (DIEA) in methylene chloride for about 5 minutes.

The first monomer is then placed at the first locations on the substrate. After irradiation, the slide is removed, treated in bulk, and then reinstalled in the flow cell.

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Alternatively, a fluid containing the first monomer, preferably also protected by a protective group, is circulated through the cavity by way of pump 116. If, for example, it is desired to attach the amino acid Y to the substrate at the first locations, the amino acid Y (bearing a protective group on its  $\alpha$ -nitrogen), along with reagents used to render the monomer reactive, and/or a carrier, is circulated from a storage container 118, through the pump, through the cavity, and back to the inlet of the pump.

The monomer carrier solution is, in a preferred embodiment, formed by mixing of a first solution (referred to herein as solution "A") and a second solution (referred to herein as solution "B"). Table 2 provides an illustration of a mixture which may be used for solution A.

TABLE 2

Representative Monomer Carrier Solution "A"
100 mg NVOC amino protected amino acid
37 mg HOBT (1-Hydroxybenzotriazole)
250 $\mu$ l DMF (Dimethylformamide)
86 $\mu$ l DIEA (Diisopropylethylamine)

The composition of solution B is illustrated in Table 3. Solutions A and B are mixed and allowed to react at room temperature for about 8 minutes, then diluted with 2 ml of DMF, and 500  $\mu$ l are applied to the surface of the slide or the solution is circulated through the reactor system and allowed to react for about 2 hours at room temperature. The slide is then washed with DMF, methylene chloride and ethanol.

TABLE 3

Representative Monomer Carrier Solution "B"
250 $\mu$ l DMF
111 mg BOP (Benzotriazolyl-n-oxy-tris(dimethylamino) phosphoniumhexafluorophosphate)

As the solution containing the monomer to be attached is circulated through the cavity, the amino acid or other monomer will react at its carboxy terminus with amino groups on the regions of the substrate which have been deprotected. Of course, while the invention is illustrated by way of circulation of the monomer through the cavity, the invention could be practiced by way of removing the slide from the reactor and submerging it in an appropriate monomer solution.

After addition of the first monomer, the solution containing the first amino acid is then purged from the system. After circulation of a sufficient amount of the DMF/methylene chloride such that removal of the amino acid can be assured (e.g., about 50X times the volume of the cavity and carrier lines), the mask or substrate is repositioned, or a new mask is utilized such that second regions on the substrate will be exposed to light and the light 124 is engaged for a second exposure. This will deprotect second regions on the substrate and the process is repeated until the desired polymer sequences have been synthesized.

The entire derivatized substrate is then exposed to a receptor of interest, preferably labeled with, for example, a fluorescent marker, by circulation of a solution or suspension of the receptor through the cavity or by contacting the surface of the slide in bulk. The receptor will preferentially bind to certain regions of the substrate which contain complementary sequences.

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Antibodies are typically suspended in what is commonly referred to as "supercocktail," which may be, for example, a solution of about 1% BSA, bovine serum albumin), 0.5% Tween® non-ionic detergent in PBS (phosphate buffered saline) buffer. The antibodies are diluted into the supercocktail buffer to a final concentration of, for example, about 0.1 to 4  $\mu$ g/ml.

FIG. 8B illustrates an alternative preferred embodiment of the reactor shown in FIG. 8A. According to this embodiment, the mask 128 is placed directly in contact with the substrate. Preferably, the etched portion of the mask is placed face down so as to reduce the effects of light dispersion. According to this embodiment, the lenses 120 and 126 are not necessary because the mask is brought into close proximity with the substrate.

For purposes of increasing the signal-to-noise ratio of the technique, some embodiments of the invention provide for exposure of the substrate to a first labeled or unlabeled receptor followed by exposure of a labeled, second receptor (e.g., an antibody) which binds at multiple sites on the first receptor. If, for example, the first receptor is an antibody derived from a first species of an animal, the second receptor is an antibody derived from a second species directed to epitopes associated with the first species. In the case of a mouse antibody, for example, fluorescently labeled goat antibody or antiserum which is antimouse may be used to bind at multiple sites on the mouse antibody, providing several times the fluorescence compared to the attachment of a single mouse antibody at each binding site. This process may be repeated again with additional antibodies (e.g., goat-mouse-goat, etc.) for further signal amplification.

In preferred embodiments an ordered sequence of masks is utilized. In some embodiments it is possible to use as few as a single mask to synthesize all of the possible polymers of a given monomer set.

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If, for example, it is desired to synthesize all 16 dinucleotides from four bases, a 1 cm square synthesis region is divided conceptually into 16 boxes, each 0.25 cm wide. Denote the four monomer units by A, B, C, and D. The first reactions are carried out in four vertical columns, each 0.25 cm wide. The first mask exposes the left-most column of boxes, where A is coupled. The second mask exposes the next column, where B is coupled; followed by a third mask, for the C column; and a final mask that exposes the right-most column, for D. The first, second, third, and fourth masks may be a single mask translated to different locations.

The process is repeated in the horizontal direction for the second unit of the dimer. This time, the masks allow exposure of horizontal rows, again 0.25 cm wide. A, B, C, and D are sequentially coupled using masks that expose horizontal fourths of the reaction area. The resulting substrate contains all 16 dinucleotides of four bases.

The eight masks used to synthesize the dinucleotide are related to one another by translation or rotation. In fact, one mask can be used in all eight steps if it is suitably rotated and translated. For example, in the example above, a mask with a single transparent region could be sequentially used to expose each of the vertical columns, translated 90°, and then sequentially used to allow exposure of the horizontal rows.

Tables 4 and 5 provide a simple computer program in Quick Basic for planning a masking program and a sample output, respectively, for the synthesis of a polymer chain of three monomers ("residues") having three different monomers in the first level, four different monomers in the second level, and five different monomers in the third level in a striped pattern. The output of the program is the number of cells, the number of "stripes" (light regions) on each mask, and the amount of translation required for each exposure of the mask.

TABLE 4  
Mask Strategy Program

```

DEFINT A-Z
DIM b(20), w(20), i(300)
FS = "LPT1"
OPEN FS FOR OUTPUT AS #1
imax = 3           'Number of residues
b(1) = 3: b(2) = 4: b(3) = 5 'Number of building blocks for res 1,2,3
g = 1: imax(1) = 1
FOR j = 1 TO imax: g = g * b(j): NEXT j
w(0) = 0: w(1) = g / b(1)
PRINT #1, "MASK2.BAS ", DATS, TIMES: PRINT #1,
PRINT #1, USING "Number of residues=##"; imax
FOR j = 1 TO imax
PRINT #1, USING "             Residue ##           ## building blocks"; j; b(j)
NEXT j
PRINT #1, " "
PRINT #1, USING "Number of cells=####"; g: PRINT #1,
FOR j = 2 TO imax
imax(j) = imax(j - 1) * b(j - 1)
w(j) = w(j - 1) / b(j)
NEXT j
FOR j = 1 TO imax
PRINT #1, USING "Mask for residue ##"; j: PRINT #1,
PRINT #1, USING "   Number of stripes=##"; imax(j)
PRINT #1, USING "   Width of each stripe=##"; w(j)
FOR i = 1 TO imax(j)
a = 1 + (i - 1) * w(j - 1)
ae = a + w(j) - 1
PRINT #1, USING " Stripe ## begins at location #### and ends at ####"; 1; a; ae
NEXT i
PRINT #1,
PRINT #1, USING " For each of ## building blocks, translate mask by ##
cell(s)"; b(j); w(j),
PRINT #1, PRINT #1, PRINT #1,

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TABLE 4-continued

Mask Strategy Program

NEXT J

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TABLE 5

Masking Strategy Output

Number of residues= 3

Residue 1 3 building blocks  
Residue 2 4 building blocks  
Residue 3 5 building blocks

Number of cells= 60

Mask for residue 1:

Number of stripes= 1  
Width of each stripe= 20  
Stripe 1 begins at location 1 and ends at 20  
For each of 3 building blocks, translate mask by 20 cell(s)

Mask for residue 2:

Number of stripes= 3  
Width of each stripe= 5  
Stripe 1 begins at location 1 and ends at 5  
Stripe 2 begins at location 21 and ends at 25  
Stripe 3 begins at location 41 and ends at 45  
For each of 4 building blocks, translate mask by 5 cell(s)

Mask for residue 3:

Number of stripes= 12  
Width of each stripe= 1  
Stripe 1 begins at location 1 and ends at 1  
Stripe 2 begins at location 6 and ends at 6  
Stripe 3 begins at location 11 and ends at 11  
Stripe 4 begins at location 16 and ends at 16  
Stripe 5 begins at location 21 and ends at 21  
Stripe 6 begins at location 26 and ends at 26  
Stripe 7 begins at location 31 and ends at 31  
Stripe 8 begins at location 36 and ends at 36  
Stripe 9 begins at location 41 and ends at 41  
Stripe 10 begins at location 46 and ends at 46  
Stripe 11 begins at location 51 and ends at 51  
Stripe 12 begins at location 56 and ends at 56  
For each of 5 building blocks, translate mask by 1 cell(s)

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#### V. Details of One Embodiment of A Fluorescent Detection Device

FIG. 9 illustrates a fluorescent detection device for detecting fluorescantly labeled receptors on a substrate. A substrate 112 is placed on an x/y translation table 202. In a preferred embodiment the x/y translation table is a model no. PM500-A1 manufactured by Newport Corporation. The x/y translation table is connected to and controlled by an appropriately programmed digital computer 204 which may be, for example, an appropriately programmed IBM PC/AT or AT compatible computer. Of course, other computer systems, special purpose hardware, or the like could readily be substituted for the AT computer used herein for illustration. Computer software for the translation and data collection functions described herein can be provided based on commercially available software including, for example, "Lab Windows" licensed by National Instruments, which is incorporated herein by reference for all purposes.

The substrate and x/y translation table are placed under a microscope 206 which includes one or more objectives 208. Light (about 488 nm) from a laser 210, which in some embodiments is a model no. 2020-05 argon ion laser manufactured by Spectraphysics, is directed at the substrate by a dichroic mirror 207 which passes greater than about 520 nm light but reflects 488 nm light. Dichroic mirror 207 may be, for example, a model no. FT510 manufactured by Carl Zeiss. Light reflected from the mirror then enters the microscope

206 which may be, for example, a model no. Axioscop 20 manufactured by Carl Zeiss. Fluorescein-marked materials on the substrate will fluoresce >488 nm light, and the fluoresced light will be collected by the microscope and passed through the mirror. The fluorescent light from the substrate is then directed through a wavelength filter 209 and, thereafter through an aperture plate 211. Wavelength filter 209 may be, for example, a model no. OG530 manufactured by Melles Griot and aperture plate 211 may be, for example, a model no. 477352/477380 manufactured by Carl Zeiss.

The fluoresced light then enters a photomultiplier tube 212 which in some embodiments is a model no. R943-02 manufactured by Hamamatsu, the signal is amplified in preamplifier 214 and photons are counted by photon counter 216. The number of photons is recorded as a function of the location in the computer 204. Pre-Amp 214 may be, for example, a model no. SR440 25 manufactured by Stanford Research Systems and photon counter 216 may be a model no. SR400 manufactured by Stanford Research Systems. The substrate is then moved to a subsequent location and the process is repeated. In preferred embodiments the data are acquired every 1 to 100  $\mu$ m with a data collection diameter of about 0.8 to 10  $\mu$ m preferred. In embodiments with sufficiently high fluorescense, a CCD (charge coupled device) detector with broadfield illumination is utilized.

35 By counting the number of photons generated in a given area in response to the laser, it is possible to determine where fluorescantly marked molecules are located on the substrate. Consequently, for a slide which has a matrix of polypeptides, for example, synthesized on the surface thereof, it is possible to determine which of the polypeptides is complementary to a fluorescantly marked receptor.

According to preferred embodiments, the intensity and duration of the light applied to the substrate is controlled by varying the laser power and scan stage rate 45 for improved signal-to-noise ratio by maximizing fluorescence emission and minimizing background noise.

While the detection apparatus has been illustrated primarily herein with regard to the detection of marked receptors, the invention will find application in other areas. For example, the detection apparatus disclosed herein could be used in the fields of catalysis, DNA or protein gel scanning, and the like.

#### VI. Determination of Relative Binding Strength of Receptors

55 The signal-to-noise ratio of the present invention is sufficiently high that not only can the presence or absence of a receptor on a ligand be detected, but also the relative binding affinity of receptors to a variety of sequences can be determined.

In practice it is found that a receptor will bind to several peptide sequences in an array, but will bind much more strongly to some sequences than others. Strong binding affinity will be evidenced herein by a strong fluorescent or radiographic signal since many receptor molecules will bind in a region of a strongly bound ligand. Conversely, a weak binding affinity will be evidenced by a weak fluorescent or radiographic

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signal due to the relatively small number of receptor molecules which bind in a particular region of a substrate having a ligand with a weak binding affinity for the receptor. Consequently, it becomes possible to determine relative binding avidity (or affinity in the case of univalent interactions) of a ligand herein by way of the intensity of a fluorescent or radiographic signal in a region containing that ligand.

Semiquantitative data on affinities might also be obtained by varying washing conditions and concentrations of the receptor. This would be done by comparison to known ligand receptor pairs, for example.

#### VII. Examples

The following examples are provided to illustrate the efficacy of the inventions herein. All operations were conducted at about ambient temperatures and pressures unless indicated to the contrary.

##### A. Slide Preparation

Before attachment of reactive groups it is preferred to clean the substrate which is, in a preferred embodiment a glass substrate such as a microscope slide or cover slip. According to one embodiment the slide is soaked in an alkaline bath consisting of, for example, 1 liter of 95% ethanol with 120 ml of water and 120 grams of sodium hydroxide for 12 hours. The slides are then washed under running water and allowed to air dry, and rinsed once with a solution of 95% ethanol.

The slides are then aminated with, for example, aminopropyltriethoxysilane for the purpose of attaching amino groups to the glass surface on linker molecules, although any omega functionalized silane could also be used for this purpose. In one embodiment 0.1% aminopropyltriethoxysilane is utilized, although solutions with concentrations from 10<sup>-7</sup>% to 10% may be used, with about 10<sup>-3</sup>% to 2% preferred. A 0.1% mixture is prepared by adding to 100 ml of a 95% ethanol/5% water mixture, 100 microliters ( $\mu$ l) of aminopropyltriethoxysilane. The mixture is agitated at about ambient temperature on a rotary shaker for about 5 minutes. 500  $\mu$ l of this mixture is then applied to the surface of one side of each cleaned slide. After 4 minutes, the slides are decanted of this solution and rinsed three times by dipping in, for example, 100% ethanol.

After the plates dry, they are placed in a 110-120° C. vacuum oven for about 20 minutes, and then allowed to cure at room temperature for about 12 hours in an argon environment. The slides are then dipped into DMF (dimethylformamide) solution, followed by a thorough washing with methylene chloride.

The aminated surface of the slide is then exposed to about 500  $\mu$ l of, for example, a 30 millimolar (mM) solution of NVOC-GABA (gamma amino butyric acid) NHS (N-hydroxysuccinimide) in DMF for attachment of a NVOC-GABA to each of the amino groups.

The surface is washed with, for example, DMF, methylene chloride, and ethanol.

Any unreacted aminopropyl silane on the surface—that is, those amino groups which have not had the NVOC-GABA attached—are now capped with acetyl groups (to prevent further reaction) by exposure to a 1:3 mixture of acetic anhydride in pyridine for 1 hour. Other materials which may perform this residual capping function include trifluoroacetic anhydride, formicacetic anhydride, or other reactive acylating agents. Finally, the slides are washed again with DMF, methylene chloride, and ethanol.

##### B. Synthesis of Eight Trimmers of "A" and "B"

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FIG. 10 illustrates a possible synthesis of the eight trimers of the two-monomer set: gly, phe (represented by "A" and "B," respectively). A glass slide bearing silane groups terminating in 6-nitroveratryloxycarboxamide (NVOC-NH) residues is prepared as a substrate. Active esters (pentafluorophenyl, OBt, etc.) of gly and phe protected at the amino group with NVOC are prepared as reagents. While not pertinent to this example, if side chain protecting groups are required for the monomer set, these must not be photoreactive at the wavelength of light used to protect the primary chain.

For a monomer set of size  $n$ ,  $n \times l$  cycles are required to synthesize all possible sequences of length  $l$ . A cycle consists of:

1. Irradiation through an appropriate mask to expose the amino groups at the sites where the next residue is to be added, with appropriate washes to remove the by-products of the deprotection.
2. Addition of a single activated and protected (with the same photochemically-removable group) monomer, which will react only at the sites addressed in step 1, with appropriate washes to remove the excess reagent from the surface.

The above cycle is repeated for each member of the monomer set until each location on the surface has been extended by one residue in one embodiment. In other embodiments, several residues are sequentially added at one location before moving on to the next location. Cycle times will generally be limited by the coupling reaction rate, now as short as 20 min in automated peptide synthesizers. This step is optionally followed by addition of a protecting group to stabilize the array for later testing. For some types of polymers (e.g., peptides), a final deprotection of the entire surface (removal of photoprotective side chain groups) may be required.

More particularly, as shown in FIG. 10A, the glass 20 is provided with regions 22, 24, 26, 28, 30, 32, 34, and 36. Regions 30, 32, 34, and 36 are masked, as shown in FIG. 10B and the glass is irradiated and exposed to a reagent containing "A" (e.g., gly), with the resulting structure shown in FIG. 10C. Thereafter, regions 22, 24, 26, and 28 are masked, the glass is irradiated (as shown in FIG. 10D) and exposed to a reagent containing "B" (e.g., phe), with the resulting structure shown in FIG. 10E. The process proceeds, consecutively masking and exposing the sections as shown until the structure shown in FIG. 10M is obtained. The glass is irradiated and the terminal groups are, optionally, capped by acetylation. As shown, all possible trimers of gly/phe are obtained.

In this example, no side chain protective group removal is necessary. If it is desired, side chain deprotection may be accomplished by treatment with ethanedithiol and trifluoroacetic acid.

In general, the number of steps needed to obtain a particular polymer chain is defined by:

 $n \times l$ 

(1)

60 where:

$n$ =the number of monomers in the basis set of monomers, and

$l$ =the number of monomer units in a polymer chain. Conversely, the synthesized number of sequences of length  $l$  will be:

 $n^l(2)$

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Of course, greater diversity is obtained by using masking strategies which will also include the synthesis of polymers having a length of less than 1. If, in the extreme case, all polymers having a length less than or equal to are synthesized, the number of polymers synthesized will be:

$$n^l + n^{l-1} + \dots + n^1. \quad (3)$$

The maximum number of lithographic steps needed will generally be  $n$  for each "layer" of monomers, i.e., the total number of masks (and, therefore, the number of lithographic steps) needed will be  $n \times l$ . The size of the transparent mask regions will vary in accordance with the area of the substrate available for synthesis and the number of sequences to be formed. In general, the size of the synthesis areas will be:

$$\text{size of synthesis areas} = (A) / (\text{Sequences})$$

where:

$A$  is the total area available for synthesis; and  
Sequences is the number of sequences desired in the area.

It will be appreciated by those of skill in the art that the above method could readily be used to simultaneously produce thousands or millions of oligomers on a substrate using the photolithographic techniques disclosed herein. Consequently, the method results in the ability to practically test large numbers of, for example, di, tri, tetra, penta, hexa, hepta, octapeptides, dodecapeptides, or larger polypeptides (or correspondingly, polynucleotides).

The above example has illustrated the method by way of a manual example. It will of course be appreciated that automated or semi-automated methods could be used. The substrate would be mounted in a flow cell for automated addition and removal of reagents, to minimize the volume of reagents needed, and to more carefully control reaction conditions. Successive masks could be applied manually or automatically.

#### C. Synthesis of a Dimer of an Aminopropyl Group and a Fluorescent Group

In synthesizing the dimer of an aminopropyl group and a fluorescent group, a functionalized durapore membrane was used as substrate. The durapore membrane was a polyvinylidene difluoride with aminopropyl groups. The aminopropyl groups were protected with the DDZ group by reaction of the carbonyl chloride with the amino groups, a reaction readily known to those of skill in the art. The surface bearing these groups was placed in a solution of THF and contacted with a mask bearing a checkerboard pattern of 1 mm opaque and transparent regions. The mask was exposed to ultraviolet light having a wavelength down to at least about 280 nm for about 5 minutes at ambient temperature, although a wide range of exposure times and temperatures may be appropriate in various embodiments of the invention. For example, in one embodiment, an exposure time of between about 1 and 5000 seconds may be used at process temperatures of between -70 and +50° C.

In one preferred embodiment, exposure times of between about 1 and 500 seconds at about ambient pressure are used. In some preferred embodiments, pressure above ambient is used to prevent evaporation.

The surface of the membrane was then washed for about 1 hour with a fluorescent label which included an active ester bound to a chelate of a lanthanide. Wash

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times will vary over a wide range of values from about a few minutes to a few hours. These materials fluoresce in the red and the green visible region. After the reaction with the active ester in the fluorophore was complete, the locations in which the fluorophore was bound could be visualized by exposing them to ultraviolet light and observing the red and the green fluorescence. It was observed that the derivatized regions of the substrate closely corresponded to the original pattern of the mask.

#### D. Demonstration of Signal Capability

Signal detection capability was demonstrated using a low-level standard fluorescent bead kit manufactured by Flow Cytometry Standards and having model no. 824. This kit includes 5.8  $\mu\text{m}$  diameter beads, each impregnated with a known number of fluorescein molecules.

One of the beads was placed in the illumination field on the scan stage as shown in FIG. 9 in a field of a laser spot which was initially shuttered. After being positioned in the illumination field, the photon detection equipment was turned on. The laser beam was unblocked and it interacted with the particle bead, which then fluoresced. Fluorescence curves of beads impregnated with 7,000 and 13,000 fluorescein molecules, are shown in FIGS. 11A and 11B respectively. On each curve, traces for beads without fluorescein molecules are also shown. These experiments were performed with 488 nm excitation, with 100  $\mu\text{W}$  of laser power. The light was focused through a 40 power 0.75 NA objective.

The fluorescence intensity in all cases started off at a high value and then decreased exponentially. The fall-off in intensity is due to photobleaching of the fluorescein molecules. The traces of beads without fluorescein molecules are used for background subtraction. The difference in the initial exponential decay between labeled and nonlabeled beads is integrated to give the total number of photon counts, and this number is related to the number of molecules per bead. Therefore, it is possible to deduce the number of photons per fluorescein molecule that can be detected. For the curves illustrated in FIGS. 11A and 11B this calculation indicates the radiation of about 40 to 50 photons per fluorescein molecule are detected.

#### E. Determination of the Number of Molecules Per Unit Area

Aminopropylated glass microscope slides prepared according to the methods discussed above were utilized in order to establish the density of labeling of the slides. The free amino termini of the slides were reacted with FITC (fluorescein isothiocyanate) which forms a covalent linkage with the amino group. The slide is then scanned to count the number of fluorescent photons generated in a region which, using the estimated 40-50 photons per fluorescent molecule, enables the calculation of the number of molecules which are on the surface per unit area.

A slide with aminopropyl silane on its surface was immersed in a 1 mM solution of FITC in DMF for 1 hour at about ambient temperature. After reaction, the slide was washed twice with DMF and then washed with ethanol, water, and then ethanol again. It was then dried and stored in the dark until it was ready to be examined.

Through, the use of curves similar to those shown in FIGS. 11A and 11B and by integrating the fluorescent

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counts under the exponentially decaying signal, the number of free amino groups on the surface after derivation was determined. It was determined that slides with labeling densities of 1 fluorescein per  $10^3 \times 10^3$  to  $\sim 2 \times 2$  nm could be reproducibly made as the concentration of aminopropyltriethoxysilane varied from  $10^{-5\%}$  to  $10^{-1\%}$ .

**F. Removal of NVOC and Attachment of A Fluorescent Marker**

NVOC-GABA groups were attached as described above. The entire surface of one slide was exposed to light so as to expose a free amino group at the end of the gamma amino butyric acid. This slide, and a duplicate which was not exposed, were then exposed to fluorescein isothiocyanate (FITC).

FIG. 12A illustrates the slide which was not exposed to light, but which was exposed to FITC. The units of the x axis are time and the units of the y axis are counts. The trace contains a certain amount of background fluorescence. The duplicate slide was exposed to 350 nm broadband illumination for about 1 minute (12 mW/cm<sup>2</sup>, ~350 nm illumination), washed and reacted with FITC. The fluorescence curves for this slide are shown in FIG. 12B. A large increase in the level of fluorescence is observed, which indicates photolysis has exposed a number of amino groups on the surface of the slides for attachment of a fluorescent marker.

**G. Use of a Mask in Removal of NVOC**

The next experiment was performed with a 0.1% aminopropylated slide. Light from a Hg-Xe arc lamp was imaged onto the substrate through a laser-ablated chrome-on-glass mask in direct contact with the substrate.

This slide was illuminated for approximately 5 minutes, with 12 mW of 350 nm broadband light and then reacted with the 1 mM FITC solution. It was put on the laser detection scanning stage and a graph was plotted as a two-dimensional representation of position color-coded for fluorescence intensity. The fluorescence intensity (in counts) as a function of location is given on 40 the color scale to the right of FIG. 13A for a mask having  $100 \times 100$   $\mu\text{m}$  squares.

The experiment was repeated a number of times through various masks. The fluorescence pattern for a 50  $\mu\text{m}$  mask is illustrated in FIG. 13B, for a 20  $\mu\text{m}$  mask 45 in FIG. 13C, and for a 10  $\mu\text{m}$  mask in FIG. 13D. The mask pattern is distinct down to at least about 10  $\mu\text{m}$  squares using this lithographic technique.

**H. Attachment of YGGFL and Subsequent Exposure to Herz Antibody and Goat Antimouse**

In order to establish that receptors to a particular polypeptide sequence would bind to a surface-bound peptide and be detected, Leu enkephalin was coupled to the surface and recognized by an antibody. A slide was derivatized with 0.1% amino propyl-triethoxysilane and protected with NVOC. A 500  $\mu\text{m}$  checkerboard mask was used to expose the slide in a flow cell using backside contact printing. The Leu enkephalin sequence (H<sub>2</sub>N-tyrosine, glycine, glycine, phenylalanine, leucine-CO<sub>2</sub>H, otherwise referred to herein as YGGFL) was attached 60 via its carboxy end to the exposed amino groups on the surface of the slide. The peptide was added in DMF solution with the BOP/HOBt/DIEA coupling reagents and recirculated through the flow cell for 2 hours at room temperature.

A first antibody, known as the Herz antibody, was applied to the surface of the slide for 45 minutes at 2  $\mu\text{g}/\text{ml}$  in a supercocktail (containing 1% BSA and 1%

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ovalbumin also in this case). A second antibody, goat anti-mouse fluorescein conjugate, was then added at 2  $\mu\text{g}/\text{ml}$  in the supercocktail buffer, and allowed to incubate for 2 hours.

An image taken at 10  $\mu\text{m}$  steps indicated that not only can deprotection be carried out in a well defined pattern, but also that (1) the method provides for successful coupling of peptides to the surface of the substrate, (2) the surface of a bound peptide is available for binding with an antibody, and (3) that the detection apparatus capabilities are sufficient to detect binding of a receptor.

**I. Monomer-by-Monomer Formation of YGGFL and Subsequent Exposure to Labeled Antibody**

Monomer-by-monomer synthesis of YGGFL and GGFL in alternate squares was performed on a slide in a checkerboard pattern and the resulting slide was exposed to the Herz antibody. This experiment and the results thereof are illustrated in FIGS. 14A, 14B, 15A and 15B.

In FIG. 14A, a slide is shown which is derivatized with the aminopropyl group, protected in this case with t-BOC (t-butoxycarbonyl). The slide was treated with TFA to remove the t-BOC protecting group. E-aminocaproic acid, which was t-BOC protected at its amino group, was then coupled onto the aminopropyl groups. The aminocaproic acid serves as a spacer between the aminopropyl group and the peptide to be synthesized. The amino end of the spacer was deprotected and coupled to NVOC-leucine. The entire slide was then illuminated with 12 mW of 325 nm broadband illumination. The slide was then coupled with NVOC-phenylalanine and washed. The entire slide was again illuminated, then coupled to NVOC-glycine and washed. The slide was again illuminated and coupled to NVOC-glycine to form the sequence shown in the last portion of FIG. 14A.

As shown in FIG. 14B, alternating regions of the slide were then illuminated using a projection print using a 500  $\times$  500  $\mu\text{m}$  checkerboard mask; thus, the amino group of glycine was exposed only in the lighted areas. When the next coupling chemistry step was carried out, NVOC-tyrosine was added, and it coupled only at those spots which had received illumination. The entire slide was then illuminated to remove all the NVOC groups, leaving a checkerboard of YGGFL in the lighted areas and in the other areas, GGFL. The Herz antibody (which recognizes the YGGFL, but not GGFL) was then added, followed by goat anti-mouse fluorescein conjugate.

The resulting fluorescence scan is shown in FIG. 15A, and the color coding for the fluorescence intensity is again given on the right. Dark areas contain the tetrapeptide GGFL, which is not recognized by the Herz antibody (and thus there is no binding of the goat anti-mouse antibody with fluorescein conjugate), and in the red areas YGGFL is present. The YGGFL pentapeptide is recognized by the Herz antibody and, therefore, there is antibody in the lighted regions for the fluorescein-conjugated goat anti-mouse to recognize.

Similar patterns are shown for a 50  $\mu\text{m}$  mask used in direct contact ("proximity print") with the substrate in FIG. 15B. Note that the pattern is more distinct and the corners of the checkerboard pattern are touching when the mask is placed in direct contact with the substrate 65 (which reflects the increase in resolution using this technique).

**J. Monomer-by-Monomer Synthesis of YGGFL and PGGFL**

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A synthesis using a 50  $\mu$ m checkerboard mask similar to that shown in FIG. 15B was conducted. However, P was added to the GGFGL sites on the substrate through an additional coupling step. P was added by exposing protected GGFGL to light and subsequent exposure to P in the manner set forth above. Therefore, half of the regions on the substrate contained YGGFL and the remaining half contained PGGFL.

The fluorescence plot for this experiment is provided in FIG. 16. As shown, the regions are again readily discernable. This experiment demonstrates that antibodies are able to recognize a specific sequence and that the recognition is not length-dependent.

#### K. Monomer-by-Monomer Synthesis of YGGFL and YPGFL

In order to further demonstrate the operability of the invention, a 50  $\mu$ m checkerboard pattern of alternating YGGFL and YPGFL was synthesized on a substrate using techniques like those set forth above. The resulting fluorescence plot is provided in FIG. 17. Again, it is seen that the antibody is clearly able to recognize the YGGFL sequence and does not bind significantly at the YPGFL regions.

#### L. Synthesis of an Array of Sixteen Different Amino Acid Sequences and Estimation of Relative Binding Affinity to Herz Antibody

Using techniques similar to those set forth above, an array of 16 different amino acid sequences (replicated four times) was synthesized on each of two glass substrates. The sequences were synthesized by attaching the sequence NVOC-GFL across the entire surface of the slides. Using a series of masks, two layers of amino acids were then selectively applied to the substrate. Each region had dimensions of 0.25 cm x 0.0625 cm. The first slide contained amino acid sequences containing only L amino acids while the second slide contained selected D amino acids. FIGS. 18A and 18B illustrate a map of the various regions on the first and second slides, respectively. The patterns shown in FIGS. 18A and 18B were duplicated four times on each slide. The slides were then exposed to the Herz antibody and fluorescein-labeled goat anti-mouse.

FIG. 19 is a fluorescence plot of the first slide, which contained only L amino acids. Red indicates strong binding (149,000 counts or more) while black indicates little or no binding of the Herz antibody (20,000 counts or less). The bottom right-hand portion of the slide appears "cut off" because the slide was broken during processing. The sequence YGGFL is clearly most strongly recognized. The sequences YAGFL and YSGFL also exhibit strong recognition of the antibody. By contrast, most of the remaining sequences show little or no binding. The four duplicate portions of the slide are extremely consistent in the amount of binding shown therein.

FIG. 20 is a fluorescence plot of the second slide. Again, strongest binding is exhibited by the YGGFL sequence. Significant binding is also detected to YaGFL, YsGFL, and YpGFL (where L-amino acids are identified by one upper case letter abbreviation, and D-amino acids are identified by one lower case letter abbreviation). The remaining sequences show less binding with the antibody. Note the low binding efficiency of the sequence yGGFL.

Table 6 lists the various sequences tested in order of relative fluorescence, which provides information regarding relative binding affinity.

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TABLE 6

Apparent Binding to Herz Ab	
L-a.a. Seq	D-a.a. Seq
YGGFL	YGGFL
YAGFL	YaGFL
YSGFL	YsGFL
LGGFL	YpGFL
FGGFL	fGGFL
YPGFL	yGGFL
LAGFL	faGFL
FAGFL	wGFL
WGGFL	yaGFL
	fpGFL
	waGFL

#### VIII. Illustrative Alternative Embodiment

According to an alternative embodiment of the invention, the methods provide for attaching to the surface a caged binding member which in its caged form has a relatively low affinity for other potentially binding species, such as receptors and specific binding substances.

According to this alternative embodiment, the invention provides methods for forming predefined regions on a surface of a solid support, wherein the predefined regions are capable of immobilizing receptors. The methods make use of caged binding members attached to the surface to enable selective activation of the predefined regions. The caged binding members are liberated to act as binding members ultimately capable of binding receptors upon selective activation of the predefined regions. The activated binding members are then used to immobilize specific molecules such as receptors on the predefined region of the surface. The above procedure is repeated at the same or different sites on the surface so as to provide a surface prepared with a plurality of regions on the surface containing, for example, the same or different receptors. When receptors immobilized in this way have a differential affinity for one or more ligands, screenings and assays for the ligands can be conducted in the regions of the surface containing the receptors.

The alternative embodiment may make use of novel caged binding members attached to the substrate. Caged (unactivated) members have a relatively low affinity for receptors of substances that specifically bind to uncaged binding members when compared with the corresponding affinities of activated binding members. Thus, the binding members are protected from reaction until a suitable source of energy is applied to the regions of the surface desired to be activated. Upon application of a suitable energy source, the caging groups libarize, thereby presenting the activated binding member. A typical energy source will be light.

Once the binding members on the surface are activated they may be attached to a receptor. The receptor chosen may be a monoclonal antibody, a nucleic acid sequence, a drug receptor, etc. The receptor will usually, though not always, be prepared so as to permit attaching it, directly or indirectly, to a binding member. For example, a specific binding substance having a strong binding affinity for the binding member and a strong affinity for the receptor or a conjugate of the receptor may be used to act as a bridge between binding members and receptors if desired. The method uses a receptor prepared such that the receptor retains its activity toward a particular ligand.

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Preferably, the caged binding member attached to the solid substrate will be a photoactivatable biotin complex, i.e., a biotin molecule that has been chemically modified with photoactivatable protecting groups so that it has a significantly reduced binding affinity for avidin or avidin analogs than does natural biotin. In a preferred embodiment, the protecting groups localized in a predefined region of the surface will be removed upon application of a suitable source of radiation to give binding members, that are biotin or a functionally analogous compound having substantially the same binding affinity for avidin or avidin analogs as does biotin.

In another preferred embodiment, avidin or an avidin analog is incubated with activated binding members on the surface until the avidin binds strongly to the binding members. The avidin so immobilized on predefined regions of the surface can then be incubated with a desired receptor or conjugate of a desired receptor. The receptor will preferably be biotinylated, e.g., a biotinylated antibody, when avidin is immobilized on the predefined regions of the surface. Alternatively, a preferred embodiment will present an avidin/biotinylated receptor complex, which has been previously prepared, to activated binding members on the surface.

#### IX. Conclusion

The present inventions provide greatly improved methods and apparatus for synthesis of polymers on substrates. It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reviewing the above description. By way of example, the invention has been described primarily with reference to the use of photoremovable protective groups, but it will be readily recognized by those of skill in the art that sources of radiation other than light could also be used. For example, in some embodiments it may be desirable to use protective groups which are sensitive to electron beam irradiation, x-ray irradiation, in combination with electron beam lithograph, or x-ray lithography techniques. Alternatively, the group could be removed by exposure to an electric current. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

What is claimed is:

1. A method of identifying at least one polypeptide for binding with a receptor comprising the sequential steps of:
  - a) generating a pattern of light and dark areas by selectively irradiating at least a first area of a surface of a substrate, said surface comprising polypeptides immobilized on said surface, said polypeptides having a photoremovable protective group, without irradiating at least a second area of said surface, to remove said protective group from said polypeptides in said first area;
  - b) simultaneously contacting said first area and said second area of said surface with a first amino acid to couple said first amino acid to said polypeptides in said first area, and not in said second area, said first amino acid having said photoremovable protective group;
  - c) generating another pattern of light and dark areas by selectively irradiating with light at least a part of said first area of said surface and at least a part of said second area to remove

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said protective group in said at least a part of said first area and said at least a part of said second area; d) simultaneously contacting said first area and said second area of said surface with a second amino acid to couple said second amino acid to said polypeptides in said at least a part of said first area and said at least a part of said second area;

- e) performing additional irradiating and amino acid contacting and coupling steps so that a matrix array of at least 100 different polypeptides is formed on said surface, each different polypeptide synthesized in an area of less than 0.1 cm<sup>2</sup>, whereby said different polypeptides have amino acid sequences and locations on said surface defined by the patterns of light and dark areas formed during the irradiating steps and the amino acids coupled in said contacting steps; and
- f) contacting said surface with a receptor and identifying which polypeptides on said surface specifically bind to said receptor.

2. The method as recited in claim 1 wherein said amino acids are selected from the group consisting of L-amino acids, D-amino acids, and synthetic amino acids.

3. The method as recited in claim 1 wherein said receptor is an antibody.

4. The method as recited in claim 1 wherein said substrate is selected from the group consisting of Langmuir Blodgett film, glass, germanium, silicon, (poly)tetrafluoroethylene, polystyrene, gallium arsenide, gallium phosphide, silicon oxide, silicon nitride, and combinations thereof.

5. The method as recited in claim 1 wherein said protective group is selected from the group consisting of 6-nitroveratryloxycarbonyl, 2-nitrobenzyloxycarbonyl, dimethoxybenzyloxycarbonyl, 5-bromo-7-nitroindolinyl,  $\alpha$ -hydroxy- $\alpha$ -methyl cinnamoyl, 2-oxymethylene antriquinone, and mixtures thereof.

6. The method as recited in claim 1 wherein each of said different polypeptides is contained within an area of between about 1  $\mu\text{m}^2$  and 10,000  $\mu\text{m}^2$ .

7. The method as recited in claim 1 wherein said steps of irradiating are carried out while said substrate is in contact with a wash solution.

8. The method as recited in claim 1 wherein said receptor further comprises a marker selected from the group consisting of radioactive markers and fluorescent markers and wherein said identifying step is a step of detecting the location on said substrate of said marker.

9. The method as recited in claim 1 wherein the irradiating step c) further comprises steps of:

- i) placing a mask adjacent to said substrate, said mask having substantially transparent regions and substantially opaque regions at a wavelength of light; and
- ii) illuminating said mask with a light source, said light source producing at least said wavelength of light, said mask permitting illumination of half of said substrate which was illuminated and half of said substrate which was not illuminated in said step a).

10. The method as recited in claim 1 wherein said additional steps are performed so as to synthesize 10<sup>3</sup> different peptides in 10<sup>3</sup> respective preselected regions on said substrate.

11. The method as recited in claim 1 wherein said additional steps are performed so as to synthesize 10<sup>6</sup>

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different peptides in  $10^6$  respective preselected regions on said substrate.

12. The method as recited in claim 1 wherein the step of identifying further comprises the step of contacting said receptor with a second receptor having a marker

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wherein said second receptor specifically binds to said receptor bound to said polypeptide.

13. The method of claim 1 wherein said additional steps are performed so that at least 1,000 different polypeptides are synthesized on said surface, and each different polypeptide is contained within an area less than about  $1 \times 10^{-3} \text{ cm}^2$ .

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<p>(54) Title: VERY LARGE SCALE IMMOBILIZED POLYMER SYNTHESIS</p> <p>(57) Abstract</p> <p>A synthetic strategy for the creation of large scale chemical diversity. Solid-phase chemistry, photolabile protecting groups, and photolithography are used to achieve light-directed spatially-addressable parallel chemical synthesis. Binary masking techniques are utilized in one embodiment. A reactor system, photoremoveable protecting groups, and improved data collection and handling techniques are also disclosed. A technique for screening linker molecules is also provided.</p> * See back of page			

## + DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

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VERY LARGE SCALE IMMOBILIZED POLYMER SYNTHESIS

This application is related to the following United States applications: U.S. Serial No. 492,462, filed March 7, 1990; U.S. Serial No. 362,901, filed June 7, 1989; U.S. Serial No. 624,120, filed December 6, 1990; U.S. Serial No. 626,730, filed December 6, 1990; and U.S. Serial No. 624,114, filed December 6, 1990. Each of these applications is incorporated herein by reference for all purposes. This application is also related to PCT application WO 90/15070 which was published December 13, 1990 and is also incorporated by reference herein for all purposes.

## BACKGROUND OF THE INVENTION

The present invention relates to the field of polymer synthesis. More specifically, the invention provides a reactor system, a masking strategy, photoremovable protecting groups, data collection and processing techniques, and applications for light directed synthesis of diverse polymer sequences on substrates.

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## SUMMARY OF THE INVENTION

Methods, apparatus, and compositions for synthesis and use of techniques for diverse polymer sequences on a substrate are disclosed, as well as applications thereof.

According to one aspect of the invention, an improved reactor system for synthesis of diverse polymer sequences on a substrate is provided. According to this embodiment the invention provides for a reactor for contacting reaction fluids to a substrate; a system for delivering selected reaction fluids to the reactor; a translation stage for moving a mask or substrate from at least a first relative location relative to a second relative location; a light for illuminating the substrate through a mask at selected times; and an appropriately programmed digital computer for selectively directing a flow of fluids from the reactor system, selectively activating the translation stage, and selectively illuminating the substrate so as to form a plurality of diverse polymer sequences on the substrate at predetermined locations.

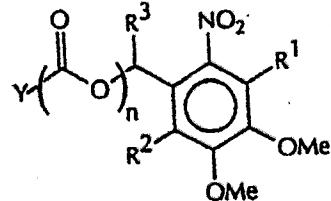
The invention also provides a technique for selection of linker molecules in VLSIPS. According to this aspect of the invention, the invention provides a method of screening a plurality of linker polymers for use in binding affinity studies. The invention includes the steps of forming a plurality of linker polymers on a substrate in selected regions, the linker polymers formed by the steps of recursively: on a surface of a substrate, irradiating a portion of the selected regions to remove a protecting group, and contacting the surface with a monomer; contacting the plurality of linker polymers with a ligand; and contacting the ligand with a labeled receptor.

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According to another aspect of the invention, improved photoremovable protecting groups are provided. According to this aspect of the invention a compound having the formula:



wherein n = 0 or 1; Y is selected from the group consisting of an oxygen of the carboxyl group of a natural or unnatural amino acid, an amino group of a natural or unnatural amino acid, or the C-5' oxygen group of a natural or unnatural deoxyribonucleic or ribonucleic acid; R<sup>1</sup> and R<sup>2</sup> independently are a hydrogen atom, a lower alkyl, aryl, benzyl, halogen, hydroxyl, alkoxy, thiol, thioether, amino, nitro, carboxyl, formate, formamido, sulfido, or phosphido group; and R<sup>3</sup> is a alkoxy, alkyl, aryl, hydrogen, or alkenyl group is provided.

The invention also provides improved masking techniques for VLSIPS. According to one aspect of the masking technique, the invention provides an ordered method for forming a plurality of polymer sequences by sequential addition of reagents comprising the step of serially protecting and deprotecting portions of the plurality of polymer sequences for addition of other portions of the polymer sequences using a binary synthesis strategy.

Improved data collection equipment and techniques are also provided. According to one embodiment, the instrumentation provides a system for determining affinity of a receptor to a ligand comprising: means for applying light to a surface of a substrate, the substrate comprising a plurality of ligands at predetermined locations, the means for

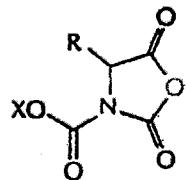
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applying directing light providing simultaneous illumination at a plurality of the predetermined locations; and an array of detectors for detecting fluorescence at the plurality of predetermined locations. The invention further provides for improved data analysis techniques including the steps of exposing fluorescently labelled receptors to a substrate, the substrate comprising a plurality of ligands in regions at known locations; at a plurality of data collection points within each of the regions, determining an amount of fluorescence from the data collection points; removing the data collection points deviating from a predetermined statistical distribution; and determining a relative binding affinity of the receptor from remaining data collection points.

Protected amino acid N-carboxy anhydrides for use in polymer synthesis are also disclosed. According to this aspect of the invention, a compound having the following formula is provided:



where R is a side chain of a natural or unnatural amino acid and X is a photoremovable protecting group.

A further understanding of the nature and advantages of the inventions herein may be realized by reference to the remaining portions of the specification and the attached drawings.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 schematically illustrates light-directed spatially-addressable parallel chemical synthesis;

Fig. 2 schematically illustrates one example of light-directed peptide synthesis;

Fig. 3 schematically illustrates the software for the automated system for synthesizing diverse polymer sequences;

Fig. 4a and 4b illustrate operation of a program for polymer synthesis;

Fig. 5 is a schematic illustration of a "pure" binary masking strategy;

Fig. 6 is a schematic illustration of a gray code binary masking strategy;

Fig. 7 is a schematic illustration of a modified gray code binary masking strategy;

Fig. 8a schematically illustrates a masking strategy for a four step synthesis;

Fig. 8b schematically illustrates synthesis of all 400 peptide dimers;

Fig. 9 is a coordinate map for the ten-step binary synthesis;

Fig. 10 schematically illustrates a data collection system;

Fig. 11 is a block diagram illustrating the architecture of the data collection system;

Fig. 12 is a flow chart illustrating operation of software for the data collection/analysis system; and

Fig. 13 schematically illustrates one example of light-directed oligonucleotide synthesis.

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## DESCRIPTION OF THE PREFERRED EMBODIMENTS

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**I. Definitions**

Certain terms used herein are intended to have the following general definitions:

1. **Complementary**: Refers to the topological compatibility or matching together of interacting surfaces of a ligand molecule and its receptor. Thus, the receptor and its ligand can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.
2. **Epitope**: The portion of an antigen molecule which is delineated by the area of interaction with the subclass of receptors known as antibodies.
3. **Ligand**: A ligand is a molecule that is recognized by a particular receptor. Examples of ligands that can be investigated by this invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones (e.g., opiates, steroids, etc.), hormone receptors, peptides, enzymes, enzyme substrates, cofactors, drugs, lectins, sugars, oligonucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.
4. **Monomer**: A member of the set of small molecules which can be joined together to form a polymer. The set of monomers includes but is not restricted to, for example, the set of common L-amino acids, the set of D-amino acids, the set of synthetic amino acids, the set of nucleotides and the set of pentoses and hexoses. As used herein, monomer refers to any member of a basis set for synthesis of a polymer. For example, dimers of the 20 naturally occurring L-amino acids form a basis set of 400

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monomers for synthesis of polypeptides. Different basis sets of monomers may be used at successive steps in the synthesis of a polymer. Furthermore, each of the sets may include protected members which are modified after synthesis.

5. Peptide: A polymer in which the monomers are alpha amino acids and which are joined together through amide bonds and is alternatively referred to as a polypeptide. In the context of this specification it should be appreciated that the amino acids may be the L-optical isomer or the D-optical isomer. Peptides are often two or more amino acid monomers long, and often more than 20 amino acid monomers long. Standard abbreviations for amino acids are used (e.g., P for proline). These abbreviations are included in Stryer, Biochemistry, Third Ed., 1988, which is incorporated herein by reference for all purposes.
6. Radiation: Energy which may be selectively applied including energy having a wavelength of between  $10^{-14}$  and  $10^4$  meters including, for example, electron beam radiation, gamma radiation, x-ray radiation, ultra-violet radiation, visible light, infrared radiation, microwave radiation, and radio waves. "Irradiation" refers to the application of radiation to a surface.
7. Receptor: A molecule that has an affinity for a given ligand. Receptors may be naturally-occurring or synthetic molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of receptors which can be employed by this invention include, but are not restricted to, antibodies,

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cell membrane receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants (such as on viruses, cells, or other materials), drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles. Receptors are sometimes referred to in the art as anti-ligands. As the term receptors is used herein, no difference in meaning is intended. A "Ligand Receptor Pair" is formed when two macromolecules have combined through molecular recognition to form a complex.

Other examples of receptors which can be investigated by this invention include but are not restricted to:

- a) Microorganism receptors: Determination of ligands which bind to receptors, such as specific transport proteins or enzymes essential to survival of microorganisms, is useful for a new class of antibiotics. Of particular value would be antibiotics against opportunistic fungi, protozoa, and those bacteria resistant to the antibiotics in current use.
- b) Enzymes: For instance, determining the binding site of enzymes such as the enzymes responsible for cleaving neurotransmitters provides useful information. Determination of ligands which bind to certain receptors to modulate the action of the enzymes which cleave the different neurotransmitters is useful in the development of drugs which can be used in the treatment of disorders of neurotransmission.
- c) Antibodies: For instance, the invention may be useful in investigating the ligand-binding site on the antibody molecule which combines with the epitope of an antigen of interest;

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determining a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for auto-immune diseases (e.g., by blocking the binding of the "self" antibodies).

d) Nucleic Acids: Sequences of nucleic acids may be synthesized to establish DNA or RNA binding sequences.

e) Catalytic Polypeptides: Polymers, preferably polypeptides, which are capable of promoting a chemical reaction involving the conversion of one or more reactants to one or more products. Such polypeptides generally include a binding site specific for at least one reactant or reaction intermediate and an active functionality proximate to the binding site, in which the functionality is capable of chemically modifying the bound reactant. Catalytic polypeptides are described in, for example, U.S. application Serial No. 404,920, which is incorporated herein by reference for all purposes.

f) Hormone receptors: For instance, the receptors for insulin and growth hormone. Determination of the ligands which bind with high affinity to a receptor is useful in the development of, for example, an oral replacement of the daily injections which diabetics must take to relieve the symptoms of diabetes, and in the other case, a replacement for the scarce human growth hormone which can only be obtained from cadavers or by recombinant DNA technology. Other examples are the vasoconstrictive hormone receptors; determination of those ligands which

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bind to a receptor may lead to the development of drugs to control blood pressure.

g) Opiate receptors: Determination of ligands which bind to the opiate receptors in the brain is useful in the development of less-addictive replacements for morphine and related drugs.

8. Substrate: A material having a rigid or semi-rigid surface. In many embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for example, wells, raised regions, etched trenches, or the like. According to other embodiments, small beads may be provided on the surface which may be released upon completion of the synthesis.

9. Protecting group: A material which is chemically bound to a monomer unit and which may be removed upon selective exposure to an activator such as electromagnetic radiation. Examples of protecting groups with utility herein include those comprising nitropiperonyl, pyrenylmethoxy-carbonyl, nitroveratryl, nitrobenzyl, dimethyl dimethoxybenzyl, 5-bromo-7-nitroindolinyl,  $\alpha$ -hydroxy- $\alpha$ -methyl cinnamoyl, and 2-oxymethylene anthraquinone.

10. Predefined Region: A predefined region is a localized area on a surface which is, was, or is intended to be activated for formation of a polymer. The predefined region may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc. For the sake of brevity herein, "predefined regions" are sometimes referred to simply as "regions."

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11. Substantially Pure: A polymer is considered to be "substantially pure" within a predefined region of a substrate when it exhibits characteristics that distinguish it from other predefined regions. Typically, purity will be measured in terms of biological activity or function as a result of uniform sequence. Such characteristics will typically be measured by way of binding with a selected ligand or receptor.
12. Activator refers to an energy source adapted to render a group active and which is directed from a source to a predefined location on a substrate. A primary illustration of an activator is light. Other examples of activators include ion beams, electric fields, magnetic fields, electron beams, x-ray, and the like.
13. Binary Synthesis Strategy refers to an ordered strategy for parallel synthesis of diverse polymer sequences by sequential addition of reagents which may be represented by a reactant matrix, and a switch matrix, the product of which is a product matrix. A reactant matrix is a  $1 \times m$  matrix of the building blocks to be added. The switch matrix is all or a subset of the binary numbers, preferably ordered, between 1 and  $m$  arranged in columns. In preferred embodiments, a binary strategy is one in which at least two successive steps illuminate half of a region of interest on the substrate. In most preferred embodiments, binary synthesis refers to a synthesis strategy which also factors a previous addition step. For example, a strategy in which a switch matrix for a masking strategy halves regions that were previously illuminated, illuminating about half of the previously illuminated region and protecting the remaining half (while also protecting

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about half of previously protected regions and illuminating about half of previously protected regions). It will be recognized that binary rounds may be interspersed with non-binary rounds and that only a portion of a substrate may be subjected to a binary scheme, but will still be considered to be a binary masking strategy within the definition herein. A binary "masking" strategy is a binary synthesis which uses light to remove protecting groups from materials for addition of other materials such as amino acids. In preferred embodiments, selected columns of the switch matrix are arranged in order of increasing binary numbers in the columns of the switch matrix.

14. Linker refers to a molecule or group of molecules attached to a substrate and spacing a synthesized polymer from the substrate for exposure/binding to a receptor.

## II. General

The present invention provides synthetic strategies and devices for the creation of large scale chemical diversity. Solid-phase chemistry, photolabile protecting groups, and photolithography are brought together to achieve light-directed spatially-addressable parallel chemical synthesis in preferred embodiments.

The invention is described herein for purposes of illustration primarily with regard to the preparation of peptides and nucleotides, but could readily be applied in the preparation of other polymers. Such polymers include, for example, both linear and cyclic polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either  $\alpha$ -,  $\beta$ -, or  $\omega$ -amino acids, heteropolymers in which a known drug is covalently bound to any of the above, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene

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sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be apparent upon review of this disclosure. It will be recognized further, that illustrations herein are primarily with reference to C- to N-terminal synthesis, but the invention could readily be applied to N- to C-terminal synthesis without departing from the scope of the invention.

#### A. Deprotection and Addition

The present invention uses a masked light source or other activator to direct the simultaneous synthesis of many different chemical compounds. Fig. 1 is a flow chart illustrating the process of forming chemical compounds according to one embodiment of the invention. Synthesis occurs on a solid support 2. A pattern of illumination through a mask 4a using a light source 6 determines which regions of the support are activated for chemical coupling. In one preferred embodiment activation is accomplished by using light to remove photolabile protecting groups from selected areas of the substrate.

After deprotection, a first of a set of building blocks (indicated by "A" in Fig. 1), each bearing a photolabile protecting group (indicated by "X") is exposed to the surface of the substrate and it reacts with regions that were addressed by light in the preceding step. The substrate is then illuminated through a second mask 4b, which activates another region for reaction with a second protected building block "B". The pattern of masks used in these illuminations and the sequence of reactants define the ultimate products and their locations, resulting in diverse sequences at predefined locations, as shown with the sequences ACEG and BDFH in the lower portion of Fig. 1. Preferred embodiments of the invention take advantage of combinatorial masking strategies to form a large number of compounds in a small number of chemical steps.

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A high degree of miniaturization is possible because the density of compounds is determined largely with regard to spatial addressability of the activator, in one case the diffraction of light. Each compound is physically accessible and its position is precisely known. Hence, the array is spatially-addressable and its interactions with other molecules can be assessed.

In a particular embodiment shown in Fig. 1, the substrate contains amino groups that are blocked with a photolabile protecting group. Amino acid sequences are made accessible for coupling to a receptor by removal of the photoprotecting groups.

When a polymer sequence to be synthesized is, for example, a polypeptide, amino groups at the ends of linkers attached to a glass substrate are derivatized with nitroveratryloxycarbonyl (NVOC), a photoremovable protecting group. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing from 2-10 monomers, diamines, diacids, amino acids, or combinations thereof. Photodeprotection is effected by illumination of the substrate through, for example, a mask wherein the pattern has transparent regions with dimensions of, for example, less than 1  $\text{cm}^2$ ,  $10^{-1} \text{ cm}^2$ ,  $10^{-2} \text{ cm}^2$ ,  $10^{-3} \text{ cm}^2$ ,  $10^{-4} \text{ cm}^2$ ,  $10^{-5} \text{ cm}^2$ ,  $10^{-6} \text{ cm}^2$ ,  $10^{-7} \text{ cm}^2$ ,  $10^{-8} \text{ cm}^2$ , or  $10^{-10} \text{ cm}^2$ . In a preferred embodiment, the regions are between about 10x10  $\mu\text{m}$  and 500x500  $\mu\text{m}$ . According to some embodiments, the masks are arranged to produce a checkerboard array of polymers, although any one of a variety of geometric configurations may be utilized.

### 1. Example

In one example of the invention, free amino groups were fluorescently labelled by treatment of the entire substrate surface with fluorescein isothiocyanate (FITC) after photodeprotection. Glass microscope slides were cleaned, aminated by treatment with

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0.1% aminopropyltriethoxysilane in 95% ethanol, and incubated at 110°C for 20 min. The aminated surface of the slide was then exposed to a 30 mM solution of the N-hydroxysuccinimide ester of NVOC-GABA (nitroveratryloxycarbonyl- $\gamma$ -amino butyric acid) in DMF. The NVOC protecting group was photolytically removed by imaging the 365 nm output from a Hg arc lamp through a chrome on glass 100  $\mu$ m checkerboard mask onto the substrate for 20 min at a power density of 12 mW/cm<sup>2</sup>. The exposed surface was then treated with 1 mM FITC in DMF. The substrate surface was scanned in an epi-fluorescence microscope (Zeiss Axioskop 20) using 488 nm excitation from an argon ion laser (Spectra-Physics model 2025). The fluorescence emission above 520 nm was detected by a cooled photomultiplier (Hamamatsu 943-02) operated in a photon counting mode. Fluorescence intensity was translated into a color display with red in the highest intensity and black in the lowest intensity areas. The presence of a high-contrast fluorescent checkerboard pattern of 100x100  $\mu$ m elements revealed that free amino groups were generated in specific regions by spatially-localized photodeprotection.

## 2. Example

Fig. 2 is a flow chart illustrating another example of the invention. Carboxy-activated NVOC-leucine was allowed to react with an aminated substrate. The carboxy activated HOBT ester of leucine and other amino acids used in this synthesis was formed by mixing 0.25 mmol of the NVOC amino protected amino acid with 37 mg HOBT (1-hydroxybenzotriazole), 111 mg BOP (benzotriazolyl-n-oxy-tris (dimethylamino)-phosphoniumhexa-fluorophosphate) and 86  $\mu$ l DIEA (diisopropylethylamine) in 2.5 ml DMF. The NVOC protecting group was removed by uniform illumination. Carboxy-activated NVOC-phenylalanine was coupled to the exposed amino groups for 2 hours at room temperature,